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14. ABSTRACT

Proliferative vitreoretinopathy (PVR) is one of the major remaining challenges in retinal surgery. PVR occurs in patients with previous complex retinal surgery and also in patients with penetrating globe injury, of which there are more than 200,000 worldwide per year. PVR is thought to result from proliferation and migration of retinal pigment epithelial (RPE) cells, leading to formation of an epiretinal membrane, retinal detachment, and loss of vision. At present, there are no reliable means of preventing this complication of ocular trauma and retinal surgery. In this project, we have addressed specific aims to [1] analyze the PVR response in wild type versus annexin A2-deficient mice, [2] define the role of A2 in the function of activated macrophages and RPE cells in PVR, and [3] examine the expression pattern of A2 in human PVR. We are now able to conclude that A2 plays a fundamental role in the pathogenesis of PVR in the mouse, that its expression is needed in both macrophages and RPE cells, and that A2 is extensively expressed within cells of epiretinal membranes in human PVR. Our data suggest that A2 may represent a "druggable" therapeutic target for the prevention of the PVR response.

15. SUBJECT TERMS

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Table of Contents

1. Introduction

Proliferative vitreoretinopathy (PVR) is a potentially blinding disorder that occurs in 8-10% of patients with primary retinal detachment, but up to 40-60% of patients with penetrating globe injury, of which there are more than 200,000 worldwide per year[1-3]. In recent studies, up to 43% of civilian and military patients with penetrating eye injuries or intraocular foreign bodies developed PVR[4-6]. In response to a retinal lesion that breaches the blood-retinal barrier, leakage of plasma proteins and circulating blood cells, possibly together with local hypoxia, conspire to stimulate retinal pigment epithelium (RPE) cells to abandon the their attachment to Bruch's membrane, proliferate, migrate, and synthesize collagens and other matrix proteins that lead to the formation of epi- and subretinal membranes with subsequent contraction[7, 8]. The early events that initiate PVR are poorly understood, but the process appears to begin with influx of CD68+ macrophage-like cells that induce delamination and proliferation of RPE cells analogous to epithelial-mesenchymal transition (EMT)[1, 9-12]. There have been few if any major advances in clinical management of PVR since its original description in 1983[13, 14].

Annexin A2 (A2) is a calcium-regulated, phospholipid-binding protein that is expressed by endothelial cells, macrophages and retinal pigment epithelial (RPE) cells. We demonstrated previously that A2 is required for the full retinal vascular proliferative response in ischemic retinopathy, based upon its ability to bind plasminogen and tissue plasminogen activator, and generate cell surface plasmin activity[15]. A2 forms a high affinity cell surface complex with its binding partner S100A10. We have also found that A2 promotes intracellular membrane repair processes that prevent inflammasome assembly and activation, and dampen pro-inflammatory responses[16]. Under the auspices of the current award, we have established that A2 is required for the full expression of PVR in the standard dispase model in mice (manuscript in preparation). These studies revealed three key findings, namely [1] that the overall PVR response is greatly attenuated or absent in the *Anxa2*$^{-/-}$ mouse, [2] that RPE cells migrate *in vitro* in response to macrophages only when both cells express A2, and [3] that cells that co-express A2 and the macrophage marker CD68, as well as cells that co-express the RPE cell marker RPE65 are abundant in preretinal membranes from human eyes exhibiting post-surgical PVR. Together, these data suggest that A2, expressed by macrophages and/or RPE cells, promotes the PVR response to retinal injury.

In this project, we have made considerable progress in each of the three aims. We are now able to conclude that A2 plays a fundamental role in the pathogenesis of PVR in the mouse, that its expression is needed in both macrophages and RPE cells, and that A2 is extensively expressed within cells of epiretinal membranes in human PVR. Current treatments for PVR are mainly surgical and largely inadequate. Our data strongly suggest that A2 represents a "druggable" therapeutic target for the prevention of the PVR response.

2. Keywords

proliferative vitreoretinopathy
annexin A2
macrophage
retinal pigmented epithelial cell
penetrating ocular injury
diabetic retinopathy
epithelial-mesenchymal transition
chronic inflammation

3. Accomplishments

Major Project Goals. In this project, we proposed three integrated specific aims with the overarching objective of understanding the potential role of the annexin A2 system in the pathogenesis of proliferative vitreoretinopathy (PVR). Under **Aim 1**, our major goal was to analyze the functional role of annexin A2 and related molecules in the classic dispase mouse model of PVR. Under **Aim 2**, we sought to specify PVR-related, annexin A2-dependent interactions between RPE cells and macrophages that might promote or enable RPE cell migration to the vitreal surface of the retina during the PVR response. **Aim**

3, finally, was directed at defining the role of the annexin A2 system in the pathogenesis and progression of human PVR. **We have made significant progress in all three aims, and have identified cellular and molecular pathways that may account for the pivotal role of annexin A2 in PVR.**

Accomplishments under the Stated Goals

Specific Aim 1. To analyze the functional role of annexin A2 and related molecules in a mouse model of PVR.

Our standard model for dispase induced PVR is as follows: *AnxA2*$^{+/+}$ and *AnxA2*$^{-/-}$ mice are treated with intravitreal dispase (0.3 units/ul; PBS)[17]. The mice (3-4 months old) are anesthetized with isoflurane by nose cone and topical proparacaine HCl (0.5%) is applied to the eye. The pupil is dilated with phenylephrine (2.5%) and tropicamide (1%). Under microscopic control, dispase (3 ul) is injected via a 30-gauge 1/6 needle fitted to a Hamilton syringe. The needle is advanced into the left eye to a depth of 1 mm at the dorsal midline just anterior to the outer canthus. The right eye remains undisturbed.

On days 1, 14, 28, and 42, mice from each group were euthanized, and the eyes harvested for histologic analysis. The eye was enucleated, and a nick created in the cornea with a #11 blade prior to incubation in 4% paraformaldehyde. The cornea and lens were then removed, and the eye oriented in 30% sucrose/PBS (18h) prior to infiltration with optimal cutting temperature/sucrose (2/1:v/v) and freezing in liquid N2-cooled isopentane. Sections (5-7 um) were arrayed six per slide, and every tenth slide stained with hematoxylin and eosin (H&E) for examination for the presence of vitreous strands, neural retinal folds, epi- and subretinal membranes, and inflammatory cells. The extent of PVR development was scored using a standardized scale. In addition, sections were examined by indirect immunofluorescence microscopy, using antibodies directed against markers for macrophages (F4/80), endothelial cells (isolectin B4), pericytes (NG2), glial cells (GFAP), Muller cells (glutamine synthase), neuronal cells (NF160), retinal pigment epithelial cells (RPE 65), and photoreceptor cells (CRALBP). These analyses have allowed us to assess the cellular locations and relative expression levels of A2 and related molecules (S100A10) within the injured eye.

Major Findings:

[1] Dispase induced PVR is attenuated in the *AnxA2*$^{-/-}$ mouse. Our histologic evaluation reveals that mice with global deficiency of A2 are significantly resistant to dispase induced PVR (**Fig. 1**). Evaluation of H&E-stained ocular sections obtained at 2 (**panel A**), 4 (**panel B**), and 6 (**panel C**) weeks revealed that RPE cells fail to migrate from their original location posterior to the retina to the vitreal surface of the retina in *Anxa2*$^{-/-}$ mice. In addition, we challenged mice with low-dose dispase in order to better observe migration of RPE cells at 4 weeks (**panel D**). Sections through the injection site show that RPE cells (identified by the presence of pigment granules and expression of RPE65 antigen) begin to migrate over the surface of the *Anxa2*$^{+/+}$ retina within 2 weeks of injury. RPE cell migration is much more limited in the *Anxa2*$^{-/-}$ eye, in which RPE cells remained confined to the RPE cell layer, or delaminated but came to rest on the scleral side of the retina. By six weeks, there is considerable distortion, detachment, and contraction of the retina often beneath the ocular lens, in the *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$, eye. Large numbers of pigmented, RPE cells can be seen streaming through the retinal wound, over the vitreal surface of the retina, and within the developing retinal scar in the *Anxa2*$^{+/+}$ eye.

[2] Quantitative histology confirms the importance of A2 in PVR. By examining of 7-11 *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ animals, each at 2, 4, and 6 weeks, we have quantified the degree of retinal disruption and RPE cell migration using a standardized scoring system (**Fig. 2**). Ten sections at or near the injection site were evaluated by trained, masked observers, and scored according to a standard rubric encompassing the degree of retinal detachment, disorganization of retinal cell layers, and epiretinal membrane formation (**A**). In addition, RPE cell migration was evaluated in a separate scoring system based on the presence of RPE cells within the retina, over the vitreal surface of the retina, or into extraretinal tissues (**B**). Previously available scoring systems in animal models have been restricted to observations made by direct ophthalmoscopic examination and/or fundus photography[17-20]. To our knowledge, histologic scoring paradigms have not been published for the mouse dispase system. At all time points examined (2, 4, and 6, weeks), *Anxa2*$^{+/+}$ mice display significantly more extensive retinal detachment, cellular disorganization and epiretinal membrane formation compared with *Anxa2*$^{-/-}$ mice (p values $\leq$ 0.006).

[3] A2-positive RPE cells migrate over the surface of the retina in PVR. In addition, we have used immunofluorescence staining to document the migration of A2-positive cells from the RPE to the surface of the retina in PVR (**Fig. 3**). We have also detected a few RPE65-positive cells on the surface of the retina during PVR. This result differs somewhat from data reported by Canto Soler et al., in which RPE65-positive cells were reported to be restricted to subretinal membranes in a dispase (0.2-0.4 u/ul)-induced PVR model in the mouse[17]. In addition, we found extensive SMA-positive staining by immunofluorescence (fibroblasts) within epiretinal membranes in the injured *Anxa2*$^{+/+}$, but not *Anxa2*$^{-/-}$ eye (**Fig. 4: 24 hours A and B, 6 weeks C and D**), and also extensive collagen deposition by Masson's trichrome staining (**4E**).

[4] A2 expression is required for recruitment of bone marrow macrophage precursors to the dispase-injected eye. In the last year, we have conducted bone marrow transplant studies to determine the origin of macrophages recruited to the eye following dispase injury (**Fig. 5**). Mice were lethally irradiated and immediately rescued with a transplant of bone marrow from *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ mice, each bearing the LacZ marker gene knocked into the A2 locus (*Anxa2*$^{LacZ/+}$ or *Anxa2*$^{LacZ/-}$ mice). In this experiment, *Anxa2*$^{LacZ/+}$ refers to mice that express LacZ under the A2 promoter, and also have a functioning A2 allele, whereas *Anxa2*$^{LacZ/-}$ mice express LacZ under the A2 promoter, but have no expression of A2 itself. One month after transplant, the mice underwent injection of intravitreal dispase to induce PVR.

When either *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ were reconstituted with *Anxa2*$^{LacZ/+}$ bone marrow, and then subjected to dispase injection, cells doubly positive for both LacZ (as indicated by positive Xgal staining) and F4/80, a macrophage marker, were present in the injured retina at 48 hours after receiving intravitreal dispase. In fact, approximately 90% of F4/80-positive cells were also Xgal-positive. These data indicated that macrophages recruited to the injured retina came from the bone marrow. In contrast, Ly6G-positive cells (neutrophils) were also present, but did not co-expressed Xgal; this result was expected since neutrophils express significant amounts of A2[21]. Notably, when irradiated *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ mice were reconstituted with *Anxa2*$^{LacZ/-}$ bone marrow, fewer F4/80-positive cells were present in and around the injured retina, and none expressed Xgal. Ly6G-positive cells did infiltrate the retina, but none expressed Xgal. These data strongly suggest that A2 is required for recruitment of macrophage precursors from the bone marrow to the injured eye.

Based upon these data, we conclude that dispase injection results in a dramatic, chronic inflammatory response in the normal mouse eye. This response appears to be characterized by A2-dependent recruitment of bone marrow-derived macrophage precursors into the injected eye. Recruitment of macrophages is followed by migration of A2-positive cells through the retinal wound and over the vitreal surface of the retina. Ultimately, there is extensive deposition of collagen, contraction of the epiretinal membrane, and retinal detachment, leading to severe disorganization and destruction of the normal retina and adjacent structures. We show for the first time, that the absence of annexin A2 is associated with reduced recruitment of bone marrow-derived macrophages and a reduced PVR response. Together, these data suggest that A2 may actively promote recruitment of macrophages, as well as delamination and migration of RPE cells, giving rise to the typical PVR response. The data suggest further that A2 could represent a "druggable" therapeutic target.

[5] Antibody blockade studies. We have carried out preliminary studies to gauge the effect of anti-A2 antibodies in preventing PVR. We have developed macrophage and RPE cell PVR models (**Fig. 6C** and **D**). We co-injected *Anxa2*$^{+/+}$ and *Anxa2*$^{-/-}$ mice intravitreally with human non-pigmented ARPE-19 retinal pigment epithelial cells (50,000 per eye in 1 ul volume) together with either anti-A2 antibody (1A7) or control (1D4). Interestingly, injection site histology revealed that, in 3 out of 3 animals, there was robust migration of pigmented host RPE cells through the retinal wound in non-injected and 1D4-injected mice (compare **Fig. 6F** and **6D**). On the other hand, mice that received the anti-A2 antibody (1A7) showed healing of the retinal wound at 4 weeks, and no migration of RPE cells over the surface of the retina (compare **Fig. 6E** and **6D**). These data suggest that blockade of A2 may be useful in preventing PVR.

Specific Aim 2: To specify PVR-related, annexin A2-dependent interactions between RPE cells and macrophages.

Major Findings:

[1] In *in vitro* macrophage-induced RPE cell migration, A2 expression is required in both cell types. In this experiment, *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ RPE cells were seeded on laminin-coated, 2-micron pore filters within the upper chambers of Transwell assemblies (**Fig. 7**). Lower wells contained a variety of test substances, including RPE cell medium, macrophage medium, macrophage-conditioned medium, *Anxa2*$^{+/+}$ macrophages, or *Anxa2*$^{-/-}$ macrophages. Macrophages were either peritoneal resident (**Fig. 7A**) or bone marrow-derived (**Fig. 7B**). After 18 hours, we enumerated RPE cells that had migrated to the under surface of the filter, by first removing residual cells from the upper side of the filter, and then staining the underside with crystal violet, and quantifying RPE cells using NIH Elements software. We observed significant migration of RPE cells across the filter only when both the RPE cells and macrophages were of the *Anxa2*$^{+/+}$ genotype. This experiment indicated that A2 is essential for macrophage-induced RPE migration, and must be expressed in both cell types.

[2] Macrophage inflammatory proteins are elevated in the *Anxa2*$^{+/+}$ but not *Anxa2*$^{-/-}$ retina in PVR.

To further understand the mechanism by which macrophages might recruit RPE cells, we conducted cytokine profiling experiments on pooled retinal homogenates from *Anxa2*$^{+/+}$ and *Anxa2*$^{-/-}$ PVR or non-injected eyes (**Fig. 8**). At 2 days or 2 weeks, we found no change in a series of immunomodulatory molecules, including proinflammatory cytokines (IL-1β, IL-6, TNFα, RANTES), an immunostimulatory cytokine (INFγ), or an anti-inflammatory cytokine (IL-10) in either *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ dispase injected eyes. In contrast, we noted a 6-fold increase over non-injected controls in the level of chemokine CCL3 (aka macrophage inflammatory protein-1α, MIP-1α) in *Anxa2*$^{+/+}$ retinas at 48 hours, as well as a doubling in CCL4 (aka MIP-1β) at the same time point. In contrast, *Anxa2*$^{-/-}$ retinas showed no increase at all in either CCL3 or CCL4.

CCL3 is a member of the "CC" chemokine, or chemotactic cytokine, family of molecules that attract mononuclear cells to sites of chronic inflammation[22, 23]. CCL3 is produced by inflamed tissue and interacts with G-protein coupled receptors, CCR1 and CCR5, on monocytes and macrophages that express CCR1 or CCR5. In the subretinal fluid and vitreous humor of human eyes exhibiting PVR, CCL3 levels, along with those of many other cytokines and chemokines, have been reported to be significantly higher than in control eyes or those with rhegmatogenous retinal detachment without PVR[24, 25]. Exactly how A2 might contribute to the production of CCL3, however, remains to be determined. One possibility is that production of CCLs produced simply reflects the number of macrophages present based upon differential recruitment in the *Anxa2*$^{+/+}$ versus *Anxa2*$^{-/-}$ mouse. Another possibility is that A2 plays a direct role in the production of these chemokines. Third, it is possible that CCLs are produced upon activation of RPE cells, which is less robust in the *Anxa2*$^{-/-}$ eye.

Our data suggest that expression of these chemotactic cytokines is elevated in response to retinal injury, but only when A2 is also expressed in the retina. These data raise the question of whether macrophage-derived CCL3 and/or CCL4 might be responsible for activation and recruitment of RPE cells into and over the retinal surface during PVR. Together, our data on the cell biology of PVR suggest that expression of A2 contributes to the functional activities of both macrophages and RPE cells in PVR.

Specific Aim 3: To define the role of the annexin A2 system in the pathogenesis and progression of human PVR.

Major Findings:

[1] Human epiretinal membranes contain abundant cells that are doubly positive for A2 and RPE65, as well as cells that are doubly positive for A2 and CD68. To address the potential role of A2 in human PVR, we have been fortunate to be able to collect a series of epiretinal membrane samples from a total of 4 consenting patients under an IRB-approved protocol (**Fig. 9**). In collaboration with Dr. Szilard Kiss (Director, Retina Service, Department of Ophthalmology, Weill Cornell), samples removed from patients undergoing retinal surgery were acquired and immediately subjected to preparation for staining.

Samples were placed on sterile MF-Millipore filters, and collected immediately by a member of Dr. Hajjar's research group. PVR membrane-containing filters were placed in tissue culture dishes, and the membrane fixed with 2% paraformaldehyde (10 min, 21°C). The membrane was then rinsed three times with PBS containing Ca^{++} and Mg^{++} and stored at 4°C for up to 22 days. For immunofluorescence staining, autofluorescence was quenched with NH_4Cl, and sections then blocked with normal donkey serum. For A2 staining, incubation with primary and secondary antibodies was done at 4°C overnight and 30 minutes at 21°C, respectively, using rabbit anti-annexin A2, followed by Cy3-labeled donkey anti-rabbit. Sections were counterstained with DAPI to visualized cell nuclei, and were imaged using Nikon 80i microscope at 200x power. H & E staining revealed epiretinal membranes to be highly cellular. Cells staining positive for either the RPE65 antigen, an RPE cell marker, or CD68, a macrophage cell marker, were readily detected (**Fig. 9B, 9C**). Cells in both groups were also positive for A2. These data indicate that expression of A2 in both RPE cells and macrophages is required for human PVR, as well as the murine disease.

Opportunities for Training

Dr. Nadia Hedhli, a postdoctoral fellow, worked on Aims 1 and 2 in this project and gained extensive experience in the dispase model of PVR, ocular histology in the mouse, isolation of murine bone marrow derived and peritoneal macrophages, and isolation of primary mouse RE cells. She is now a professor of biology at Hudson Community College in Jersey City, New Jersey.

Dissemination

A manuscript describing these findings is in preparation.

Next reporting period goals

Our final goals for this project are conduct another repeat the cell migration studies described under Aim 2, conduct another repeat of the chemokine/cytokine screening and ELSA assays, recruit several more human subjects for examination of human epiretinal membranes, and complete and submit the final manuscript.

4. Impact

We feel that our work has had a significant impact at multiple levels:

[1] Our work has provided new mechanistic insights into the cell biology of PVR.

[2] By identifying A2 as a possible drug target, we feel that the work may eventually impact the field of clinical ophthalmology.

[3] This work has significant impact for the field of annexin biology, as it offers new paradigms for the physiologic function of this protein.

[4] Regarding technologic impact, we have filed a provisional patent on the use of specific antibodies to block the action of A2 in PVR.

[5] If this work leads to the development of a new medical treatment for PVR, which is sorely lacking at present, it could offer the prospect of preserved vision to post-surgical and post-trauma patients with PVR.

5. Changes/problems

Although we were able to collect fewer than anticipated samples of human epiretinal membranes, we feel we have been able to conduct enough analyses to draw provisional conclusions.

6. Products

A manuscript describing these findings is in preparation.

7. Participants

Name	Katherine A. Hajjar, MD
Project role	PI
Researcher identifier	
Nearest person month	2
Contribution	Oversight of all aspects of the project; review of data.
Funding support	This grant

Name	Szilard Kiss, MD
Project role	Co-PI
Researcher identifier	
Nearest person month	1
Contribution	Human sample procurement; review of data.
Funding support	This grant

Name	Dena Almeida
Project role	Technician
Researcher identifier	
Nearest person month	4
Contribution	Execution of dispase model; development of cell based models; all tissue processing, staining, and documentation
Funding support	This grant

8. Quad Chart

Please see attached.

9. Appendices

Figures
Quad chart

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Annexin A2 and Proliferative Vitreoretinopathy

Log No. MR130194

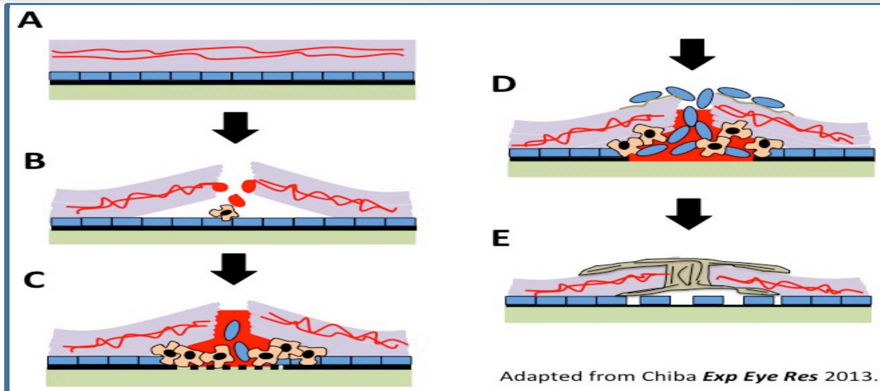
PI Katherine A. Hajjar, MD **Org:** Weill Cornell Medical College **Award Amount: $1,000,000**

Study/Product Aim(s)

- **To analyze the functional role of annexin A2 and related molecules in a mouse model of proliferative vitreoretinopathy (PVR).**
- **To specify PVR-related, annexin A2-dependent interactions between RPE cells and macrophages.**
- **To define the role of the annexin A2 system in the pathogenesis and progression of human PVR.**

Approach

This project will address the hypothesis that, in PVR, early recruitment and activity of macrophages to sites of retinal injury depends upon their expression of annexin A2. We postulate that macrophages produce proteases, growth factors, and signaling molecules that transform quiescent RPE cells into motile, fibrogenic cells that engender pre- and epiretinal scar formation, leading to further retinal damage and loss of vision.



Adapted from Chiba *Exp Eye Res* 2013.

Hypothesis: Upon retinal injury, macrophage (orange) expression of annexin A2 leads to RPE cell (blue) activation, migration, and epi/preretinal membrane scars.

We have recently established that macrophage recruitment to the hypoxic mouse retina is greatly reduced in the annexin A2-deficient mouse.

Timeline and Cost

Activities	CY	14	15	16	17
Prepare and submit application					
Aim1: Mouse model completed					
Aim 2: Co-culture studies underway					
Aim 3: Human subject studies underway					
Estimated Budget ($K)		**$000**	**$333**	**$333**	**$334**

Updated: 7/14/17

Goals/Milestones (Example)

CY14 Goal – Submit pre-application

☑ Completed

CY15 Goals – Submit full application and initiate project

☑ Establish PVR model in AnxA2$^{-/-}$, S100A10$^{-/-}$ and S100A4$^{-/-}$ mice

☑ Establish macrophage-RPE co-culture systems

☑ Initiate collection of human PVR samples

CY16 Goal – Continue experiments related to Aims 1-3

☑ Study PVR macrophage-specific A2 knockouts

☑ Continue macrophage and RPE signaling experiment

☑ Initiate human RPE cell-macrophage experiments

CY17 Goal – Complete experiments and submit manuscripts

☑ Complete bone marrow transplantation experiments

☐ Complete cytokine profiling

☐ Complete human epiretinal membrane profiling

Comments/Challenges/Issues/Concerns

Budget Expenditure to Date

Projected Expenditure: $1,000,000

Figure 1A

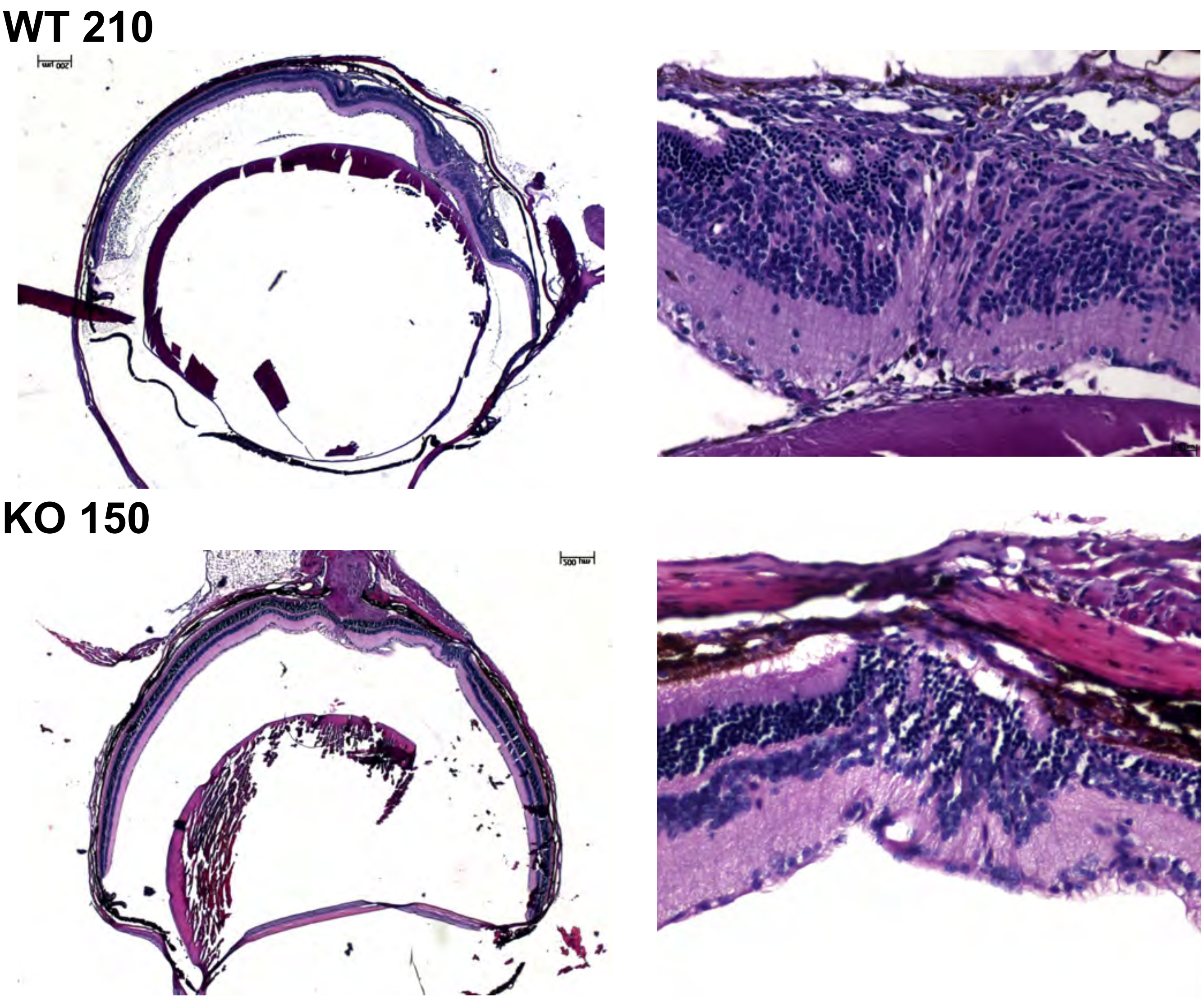


Figure 1A. C57Bl/6 mice, either *Anxa2*$^{+/+}$ (WT) or *Anxa2*$^{-/-}$ (KO) received intravitreal injections of 0.3 units/ul of dispase in the left eye.   At 2 weeks, a representative hematoxylin and eosin-stained section through a WT eye shows more extensive migration of pigmented cells from the RPE layer through the retinal wound and onto the vitreal surface of the retina in apposition to the lens. In the KO eye, migrating pigmented cells a re not detected and no epiretinal membrane has formed. Original magnification 25x left and 200x right.

Figure 1B

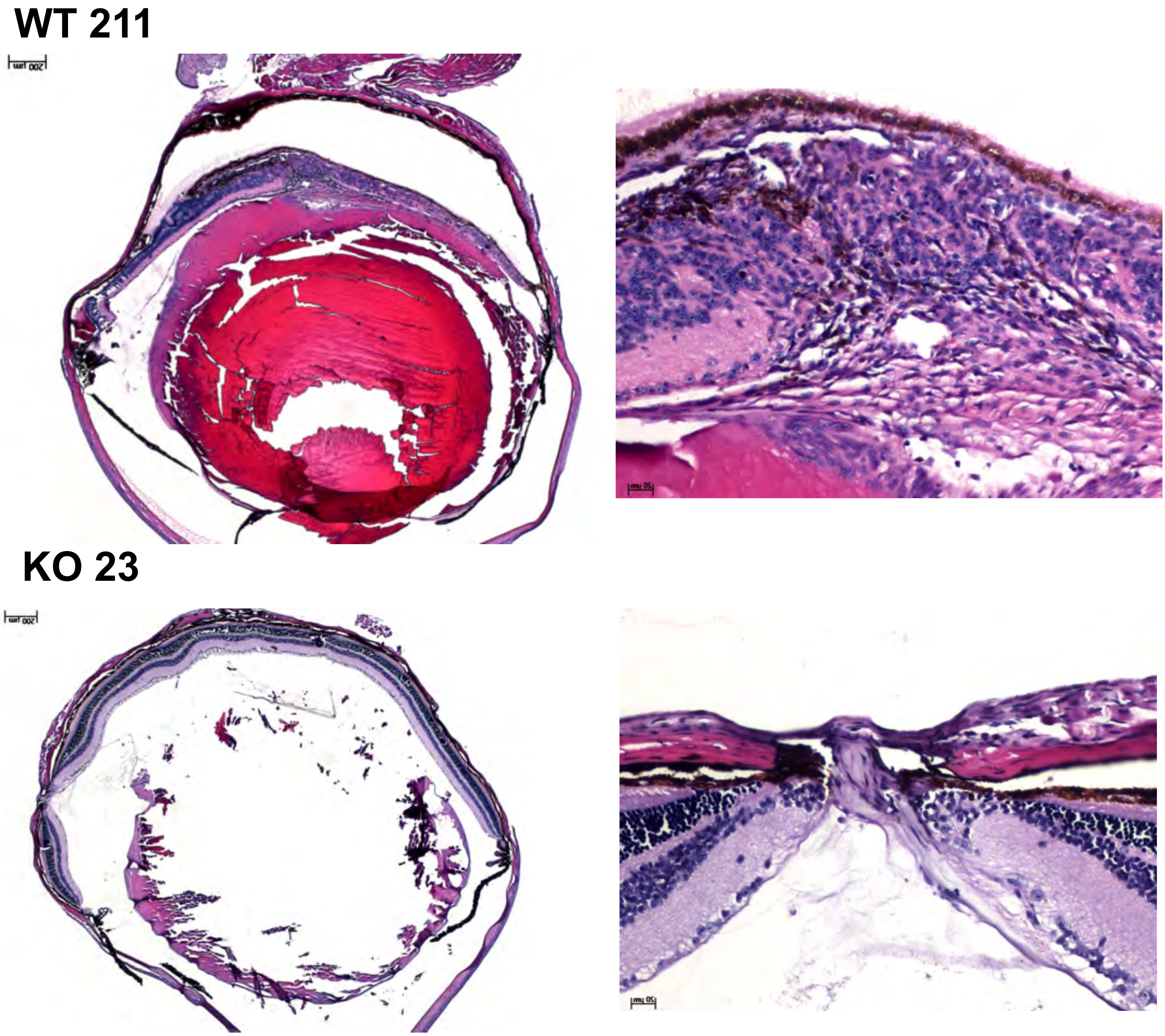


Figure 1B. At 4 weeks, a representative section from a hematoxylin and eosin stained WT section shows extensive disruption of the normal retinal architecture with invasion of pigmented cells into the retina. In the KO eye, retinal architecture is preserved with the exception of a small transretinal scar at the injection site. There is no epiretinal membrane formation. Original magnification 25x left and 200x right.

Figure 1C

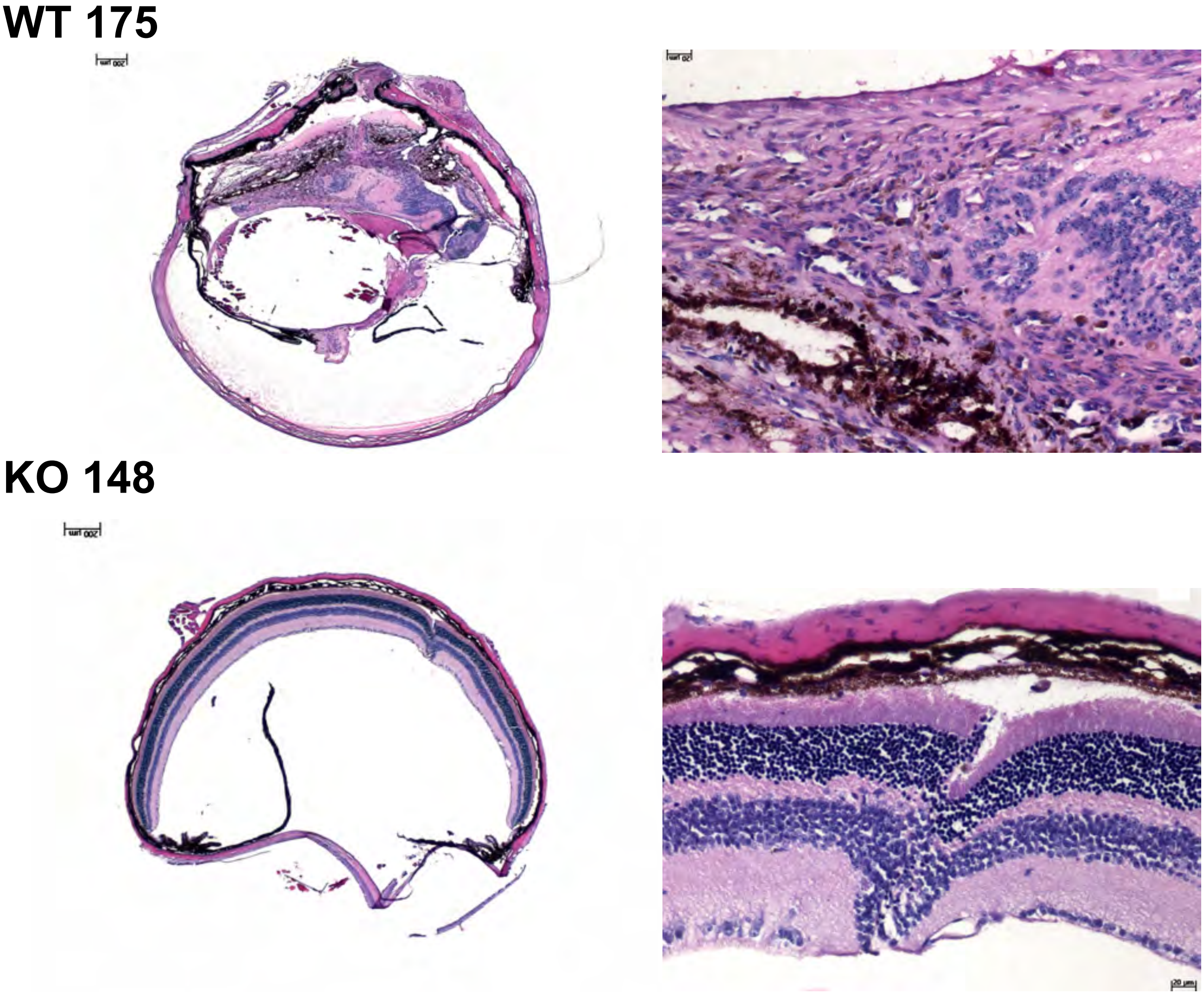


Figure 1C. At 6 weeks, there is complete detachment and disruption of the normal retinal architecture. a representative section from a WT eye shows extensive disruption of the normal retinal architecture with invasion of pigmented cells into the retina. There are numerous pigmented cells within the retinal remnant and within the epiretinal membrane. In the KO eye, retinal architecture is preserved. There are no pigmented cells within the retina, no pigmented cells within the retinal wound, and no epiretinal membrane formation. Original magnification 25x left and 200x right.

Figure 1D

WT 41

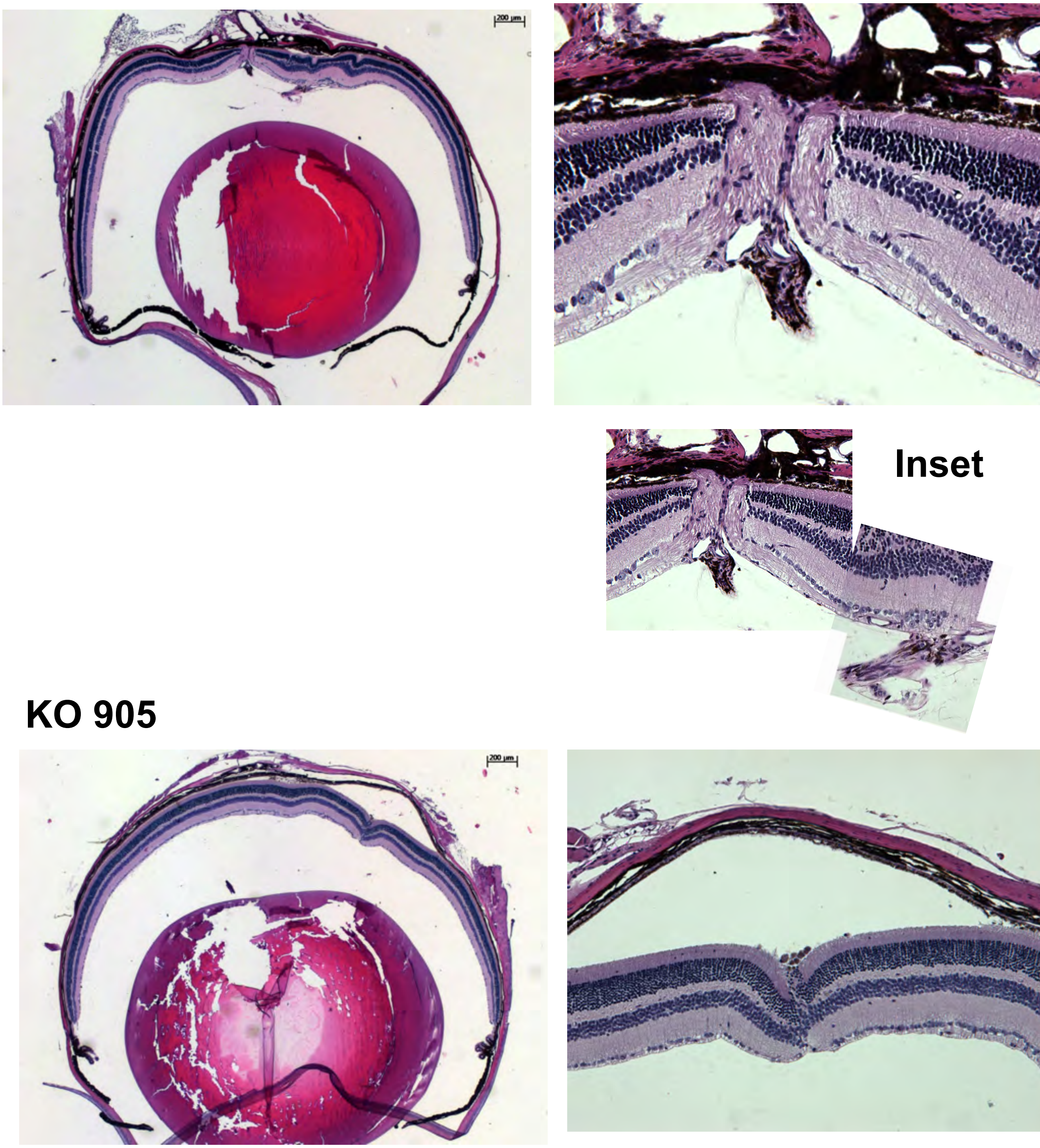


Figure 1D. To examine the effects of dispase in more detail, WT or KO mice received ultra-low intravitreal injections of dispase (0.003 units/ul). Eyes were harvested at 4 weeks, and 5-micron sections through the injection site were stained with hematoxylin and eosin. WT eyes display migration of pigmented cells, presumably RPE cells, from the RPE layer, through the injection site, and into the vitreous space. In the KO eye, a few pigmented cells are evident on the scleral surface of the retina at the injection site where the retina has separated from the RPE layer, but do not migrate through the wound onto the retinal surface. Original magnification 25x left and 200x right.

Figure 2

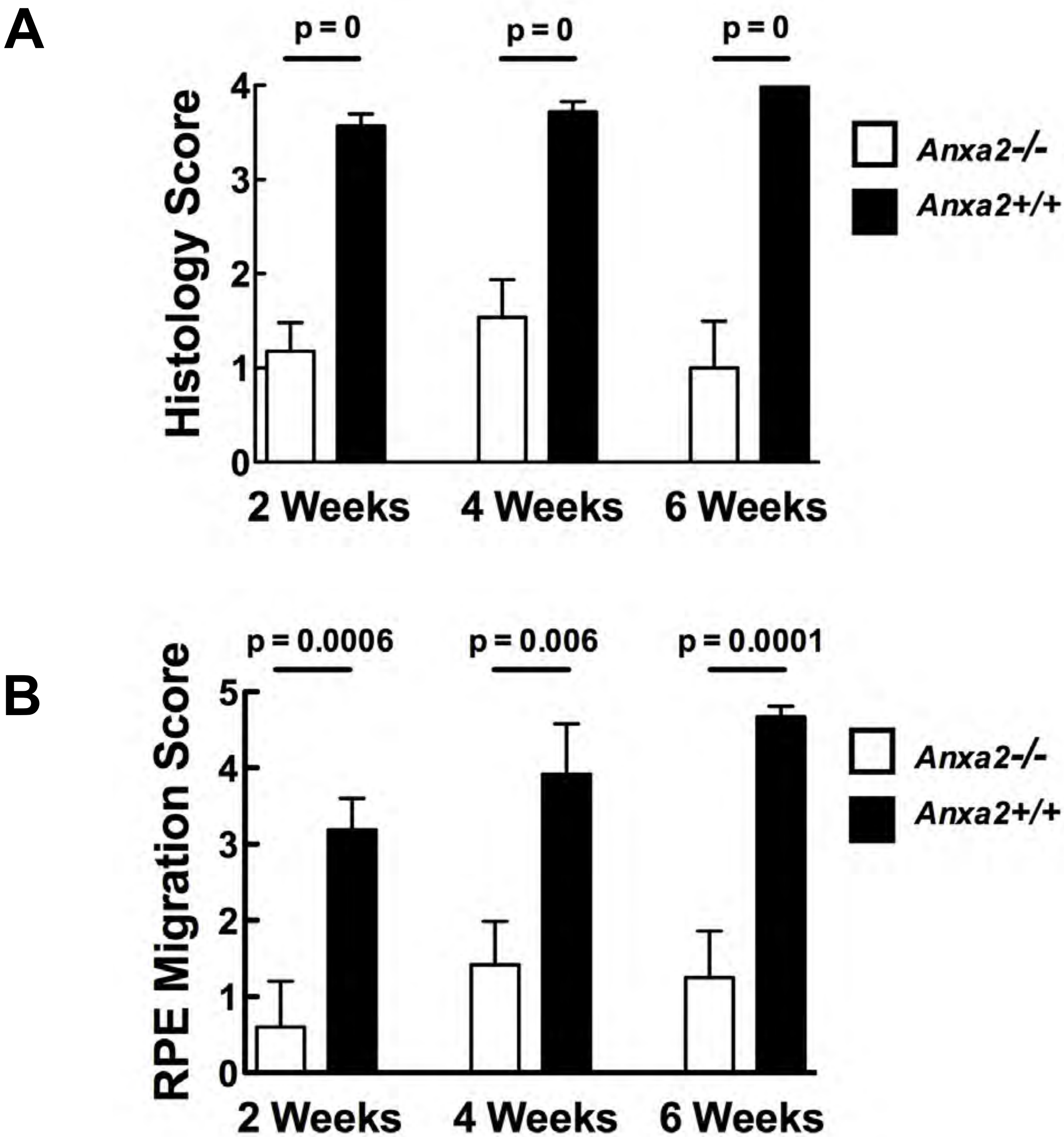


Figure 2. Hematoxylin- and eosin-stained sections (10 section per dispase-injected eye) from a total of 53 mice and were analyzed by 3 trained observers in a double-blind fashion. Sections were from six groups of mice (n=7-11 mice per group) representing *Anxa2*$^{-/-}$ or *Anxa2*$^{+/+}$ mice at 2, 4, or 6 weeks and were scored using a standard algorithm. Inter-observer variability for the 6 groups averaged 10.4 + 3.1%. The results indicate a highly significant difference in severity of PVR, as judged by overall histology score (**A**) and the extent of RPE cell migration (**B**). Scoring rubrics for overall histology and RPE cell migration are shown in panels **C** and **D**, respectively.

Figure 2

C

PVR Histology Index

Score	Retinal Detachment	Disorganization of Retinal Cell Layers	Scar Formation
0	absent	absent	absent
1	focal (<50%)	absent	absent
2	extensive (>50%)	partial (<50%)	absent
3	extensive (>50%)	extensive (>50%)	present or absent
4	extensive (>50%)	extensive (>50%)	present with cellular infiltration
5	complete destruction of retina		

D

RPE Cell Migration Index

Score	Description
0	No RPE migration
1	RPE migration limited to retina
2	RPE migration within retina and on surface of retina. Retinal cell layers remain intact.
3	RPE migration within retina and on surface of retina. Retinal cell layers at injection site are disorganized.
4	RPE migration beyond surface of retina and into extraretinal tissue.
5	Complete destruction

Figure 3

A

B

C

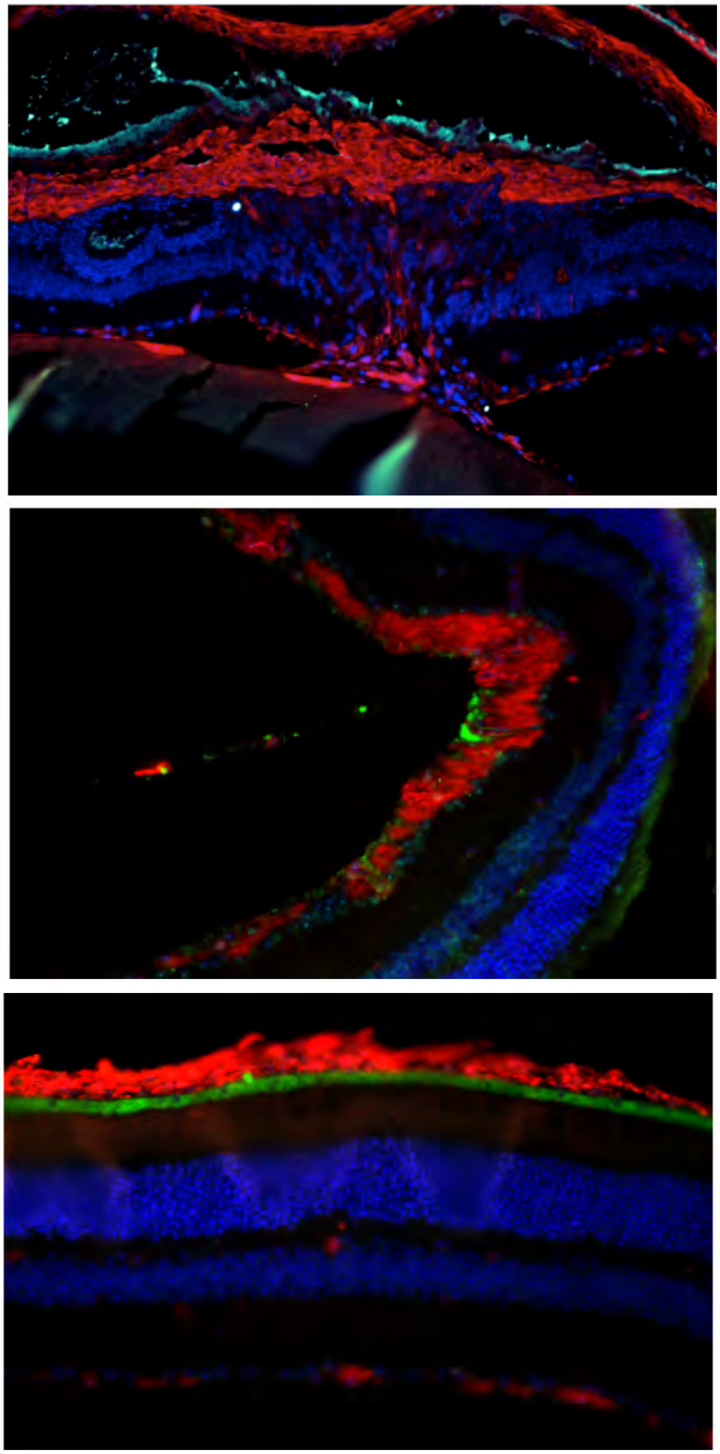


Figure 3. Immunofluorescence staining of sections of *Anxa2*$^{+/+}$ dispase-injected eyes harvested at 2 weeks. **A**. At the injection site, cells migrating from the scleral side of the retina through the retinal wound and over the vitreal surface of the retina are strongly A2-positive, but mostly negative for RPE65, a marker of quiescent RPE cells. **B.** Cells within the epiretinal membrane are strongly A2-positive, and a few are RPE-positive. **C.** In a retina without PVR, A2 staining is limited to blood vessels within the retina and the choroid. A2 (red), RPE (green), and DAPI (blue). Original magnification 200x.

Figure 4A

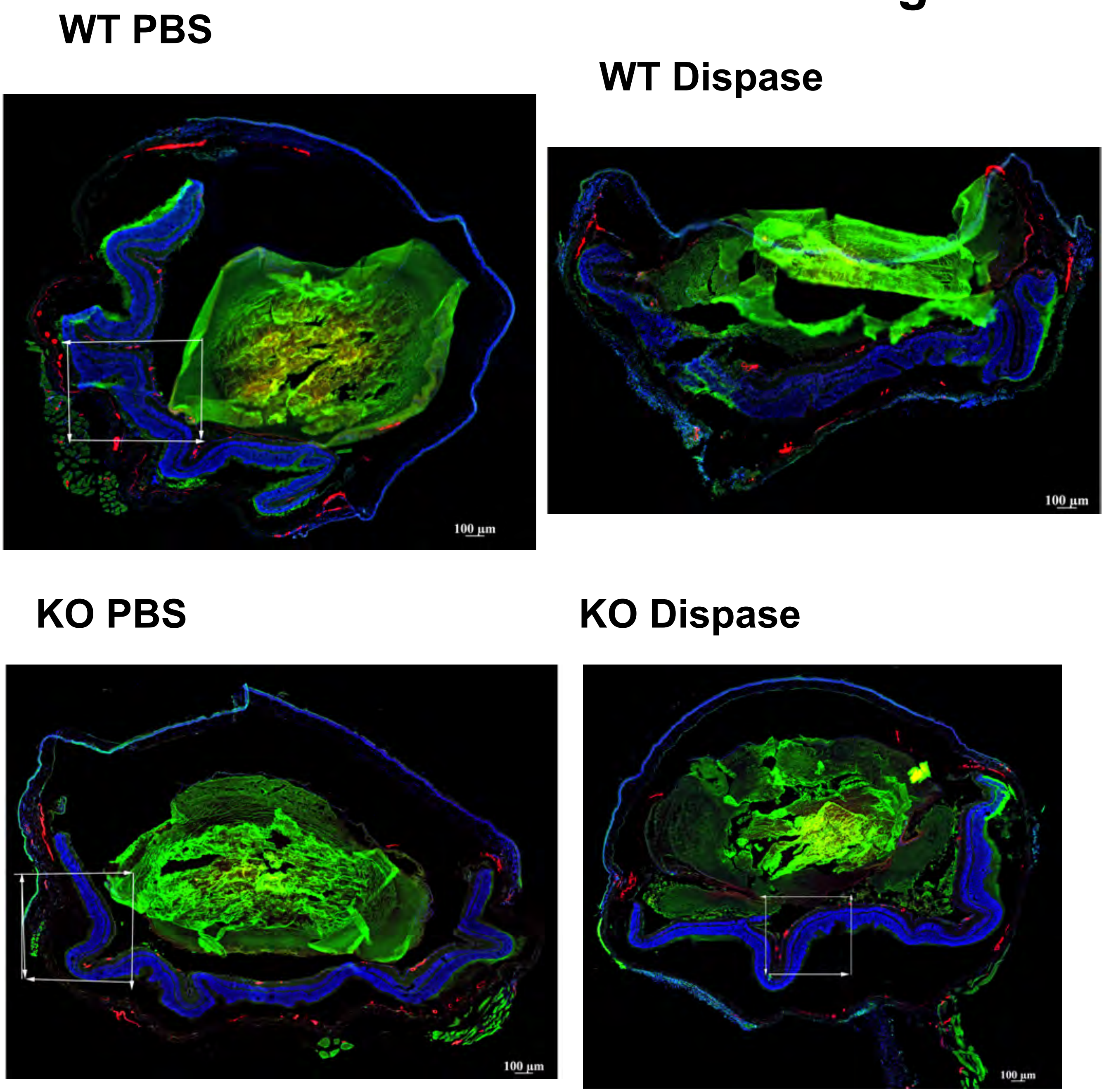


Figure 4A. Immunofluorescence staining of sections of *Anxa2*$^{+/+}$ dispase-injected eyes harvested at 24 hours. There is minimal disruption of retinal layers. Periretinal F4/80 staining is associated with dispase injection only. SMA-positive staining is mainly associated with blood vessels near the surface of the retina. Smooth muscle cell actin (red), F4/80 (green), and DAPI (blue). Original magnification 25x..

Figure 4B

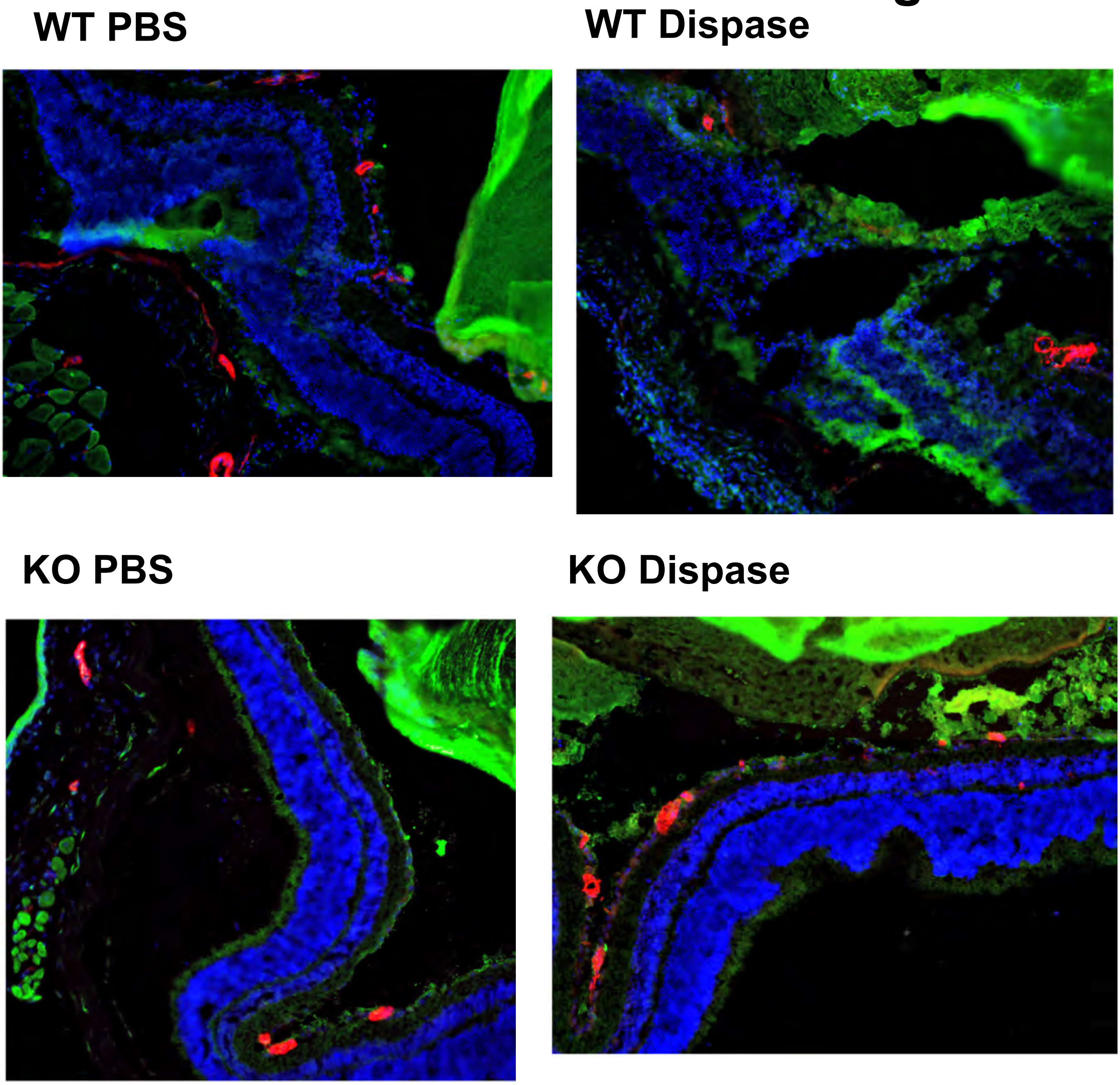


Figure 4B. Immunofluorescence staining of sections of *Anxa2*$^{+/+}$ dispase-injected eyes harvested at 24 hours – high power views. Smooth muscle cell actin (red), F4/80 (green), and DAPI (blue). Original magnification 200x.

Figure 4C

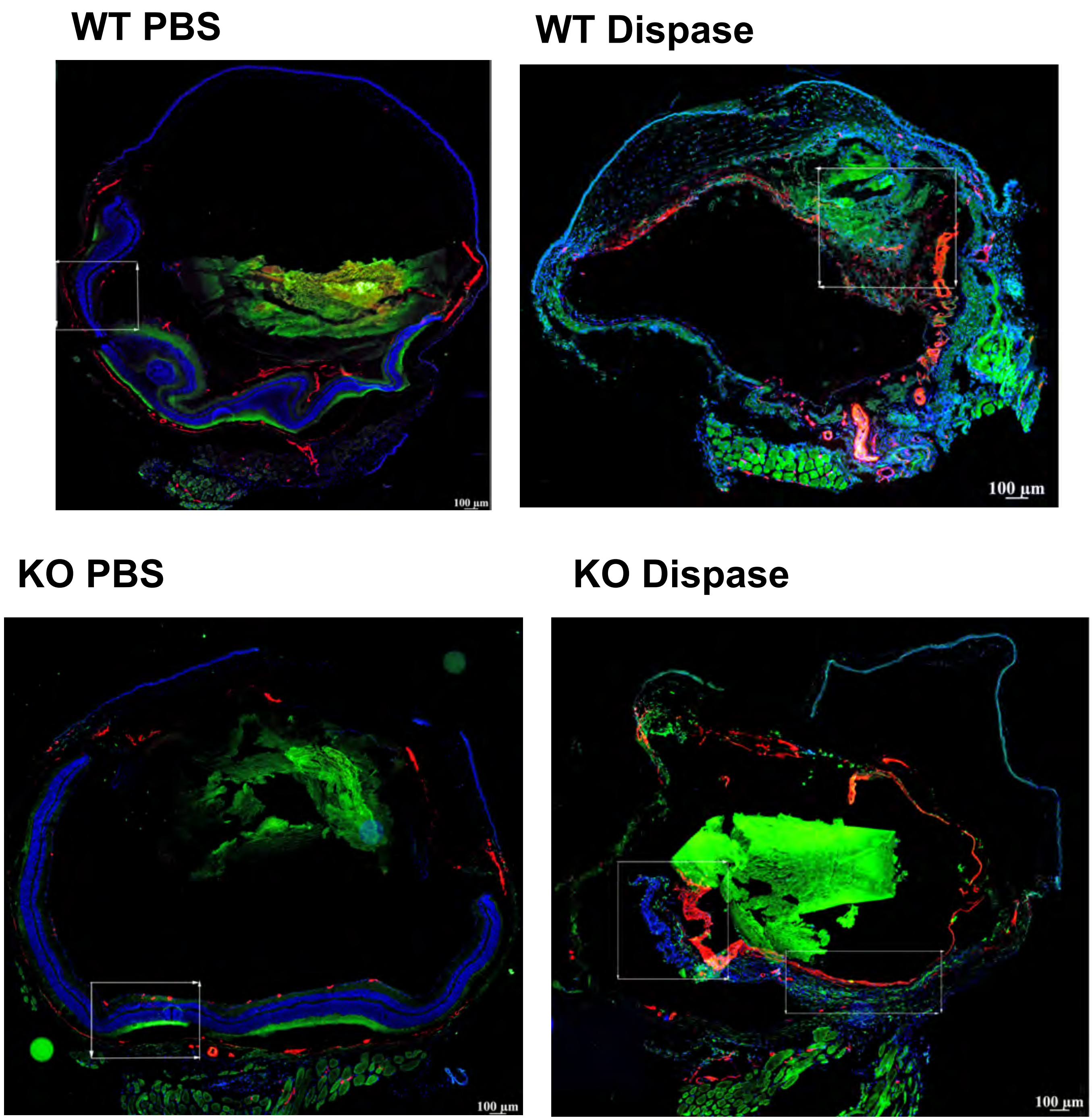


Figure 4C. Immunofluorescence staining of sections of *Anxa2*$^{+/+}$ dispase-injected eyes harvested at 6 weeks. The dispase-injected WT eye shows extreme disorganization of retinal architecture, striking epiretinal scar formation, and extensive SMA-positive staining on the vitreal surface of the retina. F4/80 staining within the retina is associated with dispase injection only. The dispase injected KO eye shows less extensive disruption of retinal architecture, no obvious scar formation, and less extensive epiretinal SMA staining. Smooth muscle cell actin (red), F4/80 (green), and DAPI (blue). Original magnification 25x.

Figure 4D

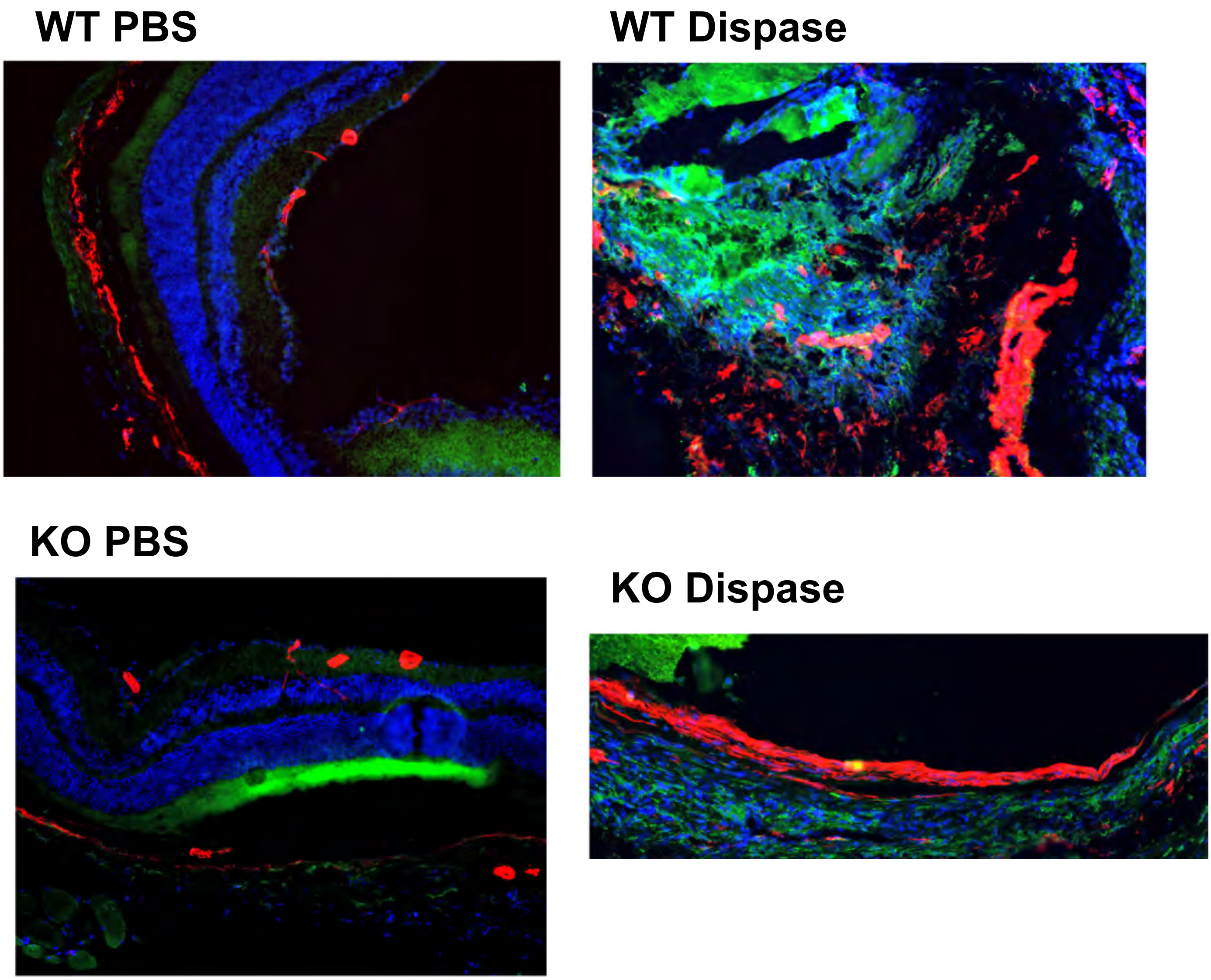
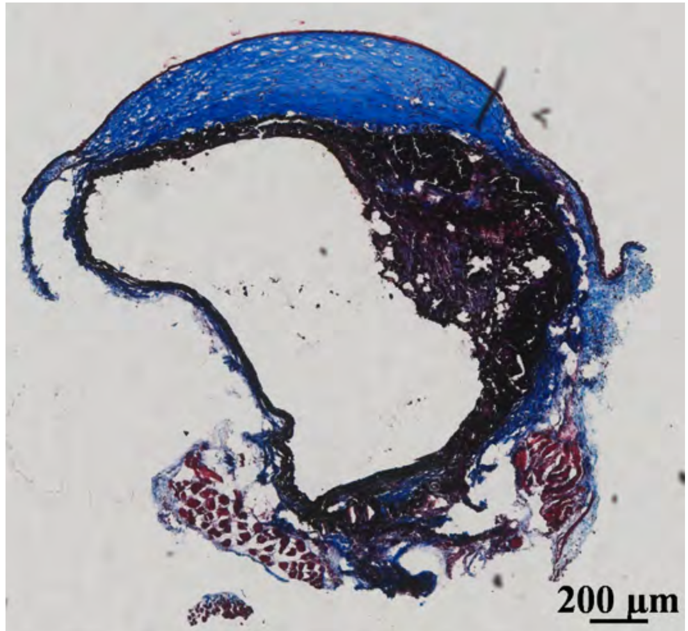


Figure 4D. Close-up views show minimal SMA-positive staining in the dispase injected KO, where retinal architecture is largely preserved compared with the dispase-injected WT eye.. Smooth muscle cell actin (red), F4/80 (green), and DAPI (blue). Original magnification 25x.

Figure 4E

WT 12



KO 15

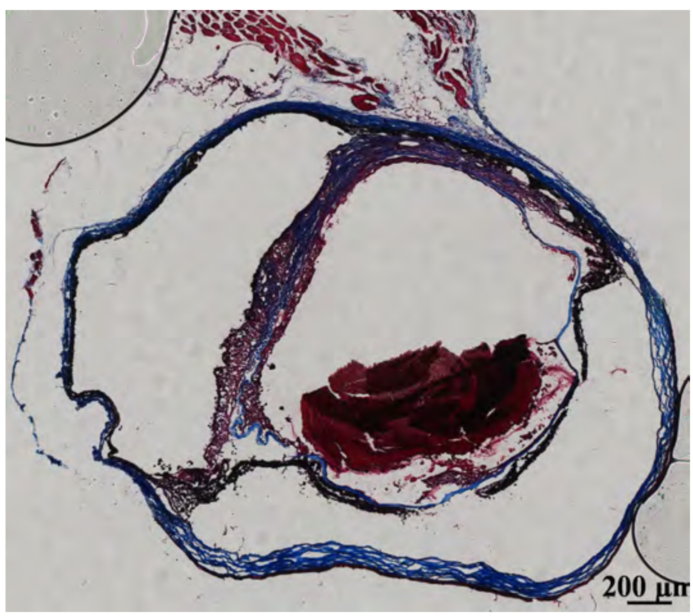
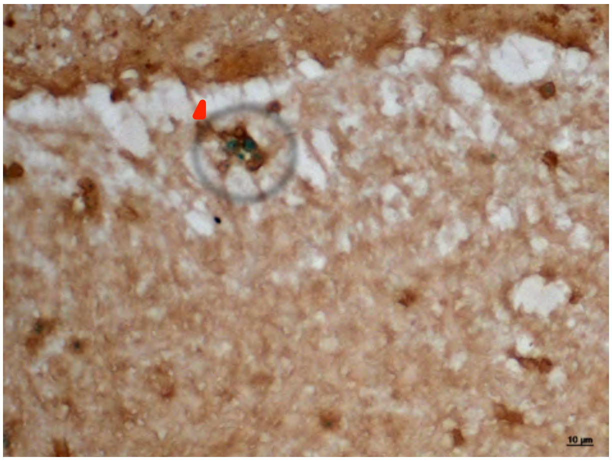


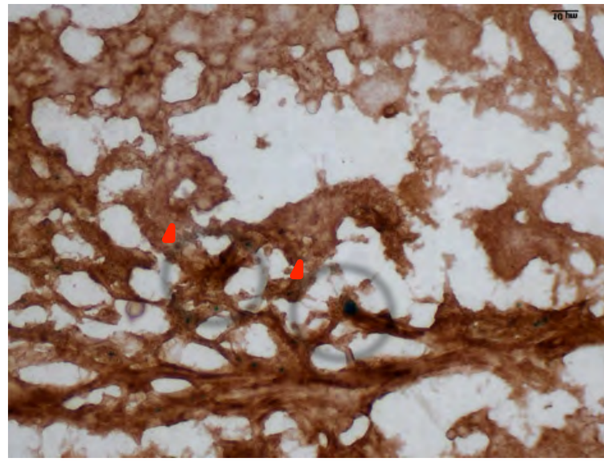
Figure 4E. Masson's trichrome stain of dispase-injected *Anxa2*$^{+/+}$ and *Anxa2*$^{-/-}$ eyes shown in panels **C** and **D**. Note extensive collagen deposition (blue) in WT eye, compared with the KO eye. Original magnification 25x.

Figure 5

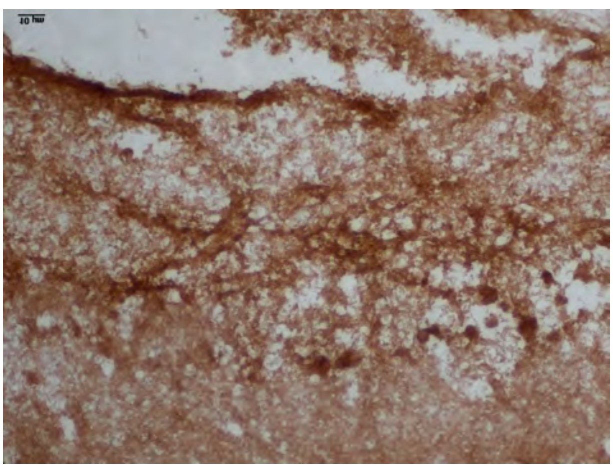
$A2^{Lacz+}$ to $A2^{+/+}$ 48hrs F4/80



$A2^{Lacz+}$ to $A2^{-/-}$ 48hrs F4/80



$A2^{Lacz-}$ to $A2^{+/+}$ 48hrs F4/80



$A2^{Lacz-}$ to $A2^{-/-}$ 48hrs F4/80

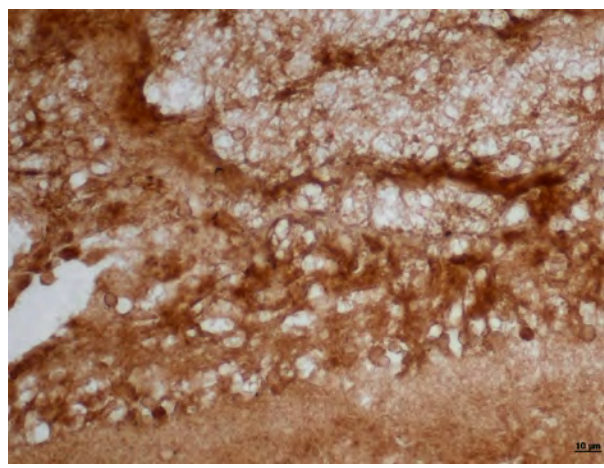


Figure 6

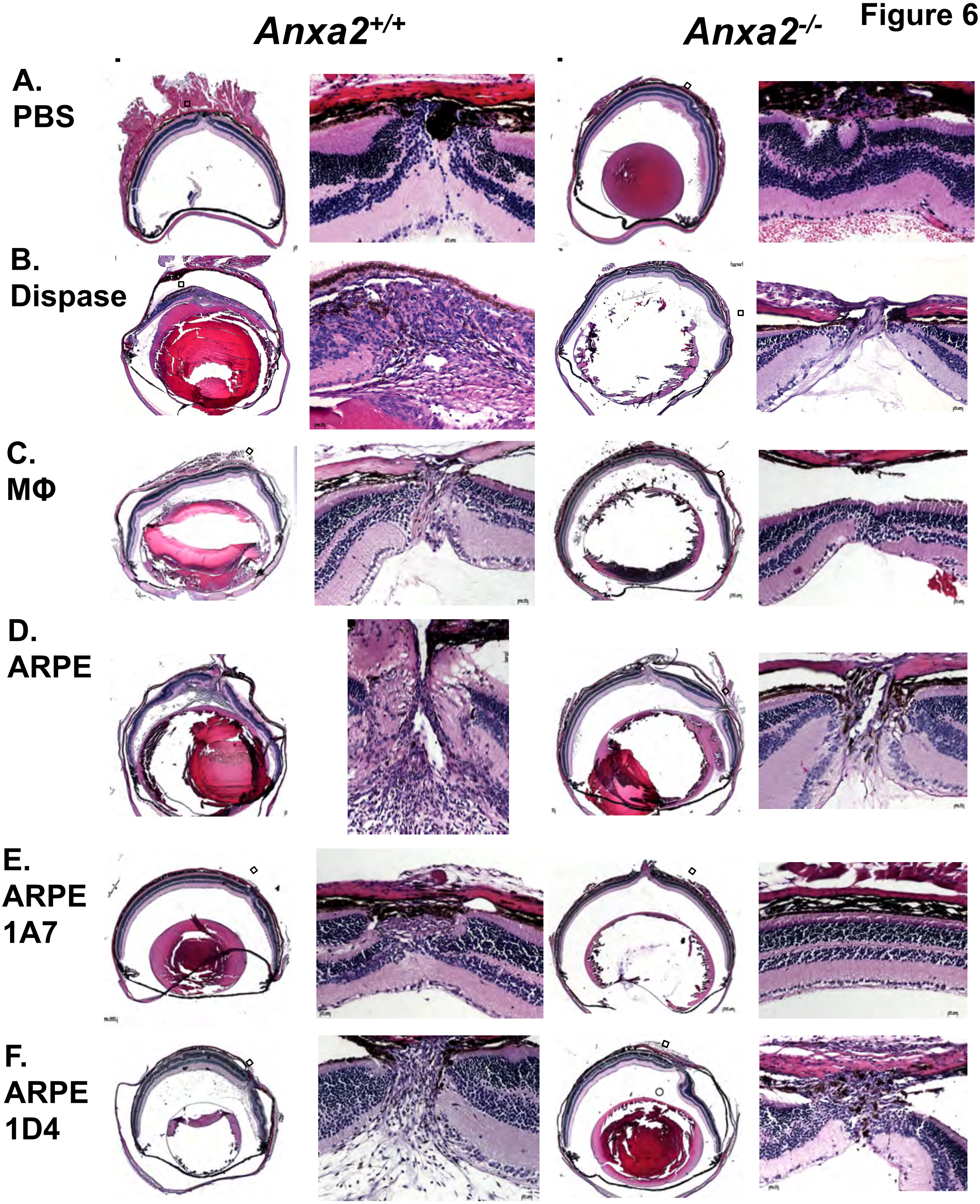


Figure 6. H and E-stained sections through injection site (box) from eye 4 weeks post induction of PVR. *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ eyes received PBS, dispase (0.3 U), 50,000 bone marrow-derived *Anxa2*$^{+/+}$ macrophages (MΦ) or 50,000 ARPE-19 cells, without or with anti-A2 antibody (1A7) or control anitbody (1D4). Original magnification 25x (whole eye) or 200x (zoomed view).

Figure 7

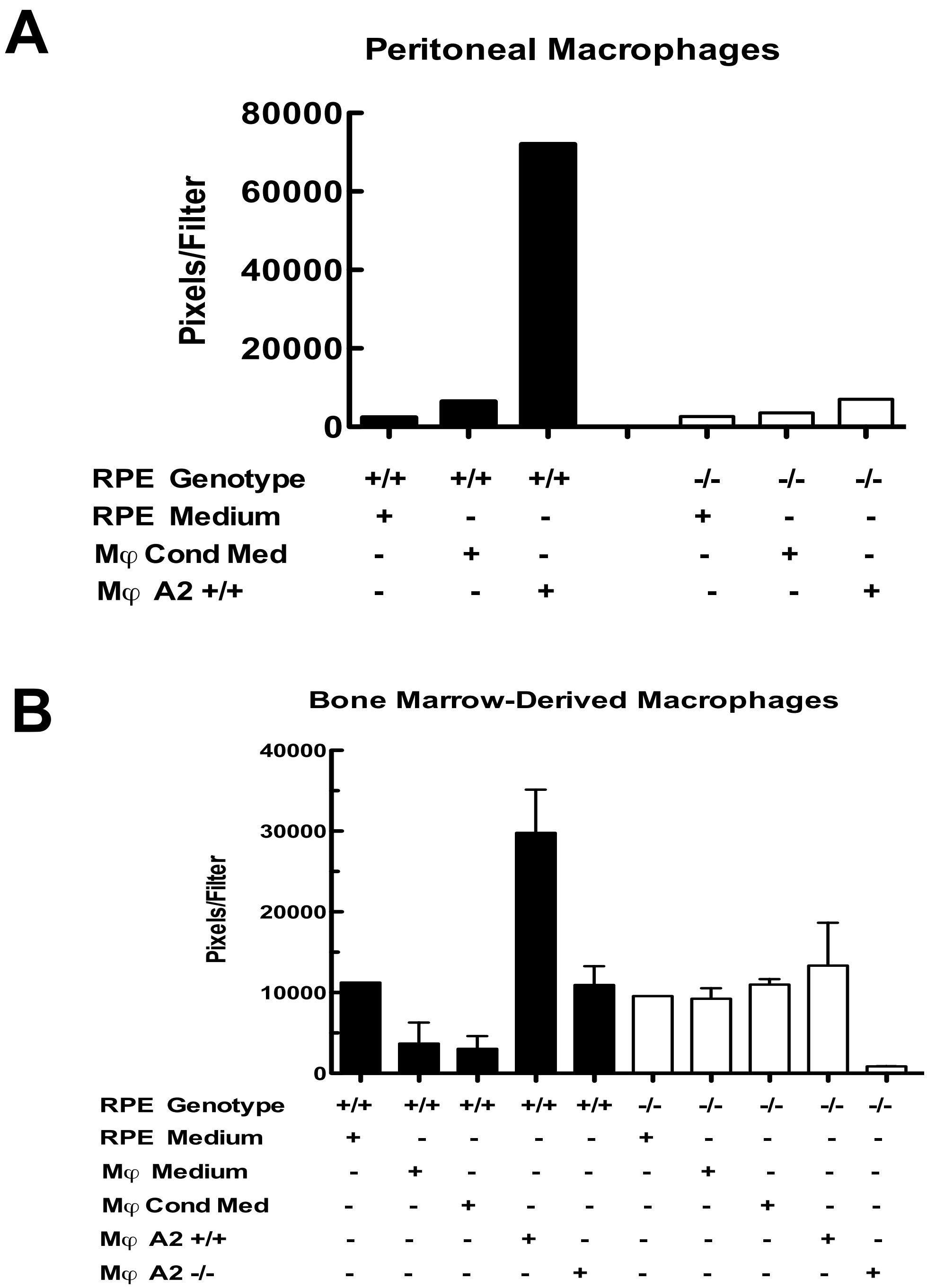


Figure 5. RPE migration assay. *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ RPE cells (1 x 10^5 cells per well) were seeded on laminin-coated, 3-micron pore, Transwell filters positioned above a lower well containing either RPE medium, macrophage medium, macrophage conditioned medium, or *Anxa2*$^{+/+}$ or *Anxa2*$^{-/-}$ peritoneal (**A**) or bone marrow-derived (**B**) macrophages (2 x 10^5 cells per well). Co-cultures were incubated for 24 hours, at which time residual RPE cells from the upper side of the filter were removed, and the underside stained with crystal violet. RPE cell migration was assessed using NIH Elements image software. Of interest, migration of RPE cells appears to depend upon both the presence of *Anxa2*$^{+/+}$ macrophages.

Figure 8

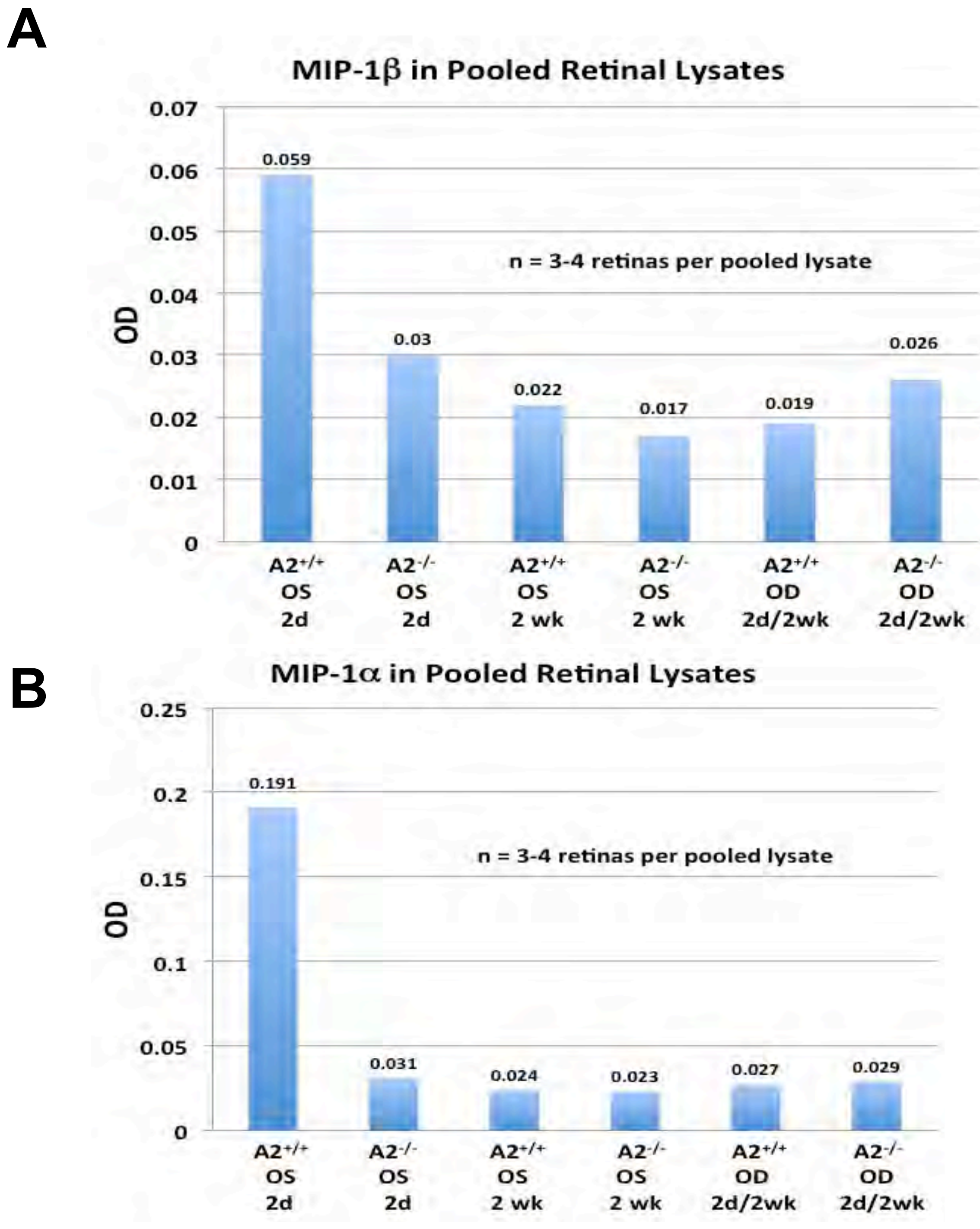


Figure 6. Dispase-injected eyes were harvested at 2 days, 2 weeks, and 4 weeks. Retinas from 3 animals per condition were pooled, and hand-homogenized at 4C in RIPA buffer in the presence of a protease inhibitor cocktail. After mixing (30 min, 4C), samples were centrifuged (15,600 x*g)* and supernatants subjected to cytokine/chemokine screening. Of a panel of 8 cyto/chemokines, only 2 displayed a difference between WT and KO. Note several fold increase in MIP-1α and MIP-1β in WT, but not in KO 2 days after dispase injection.

Figure 9A

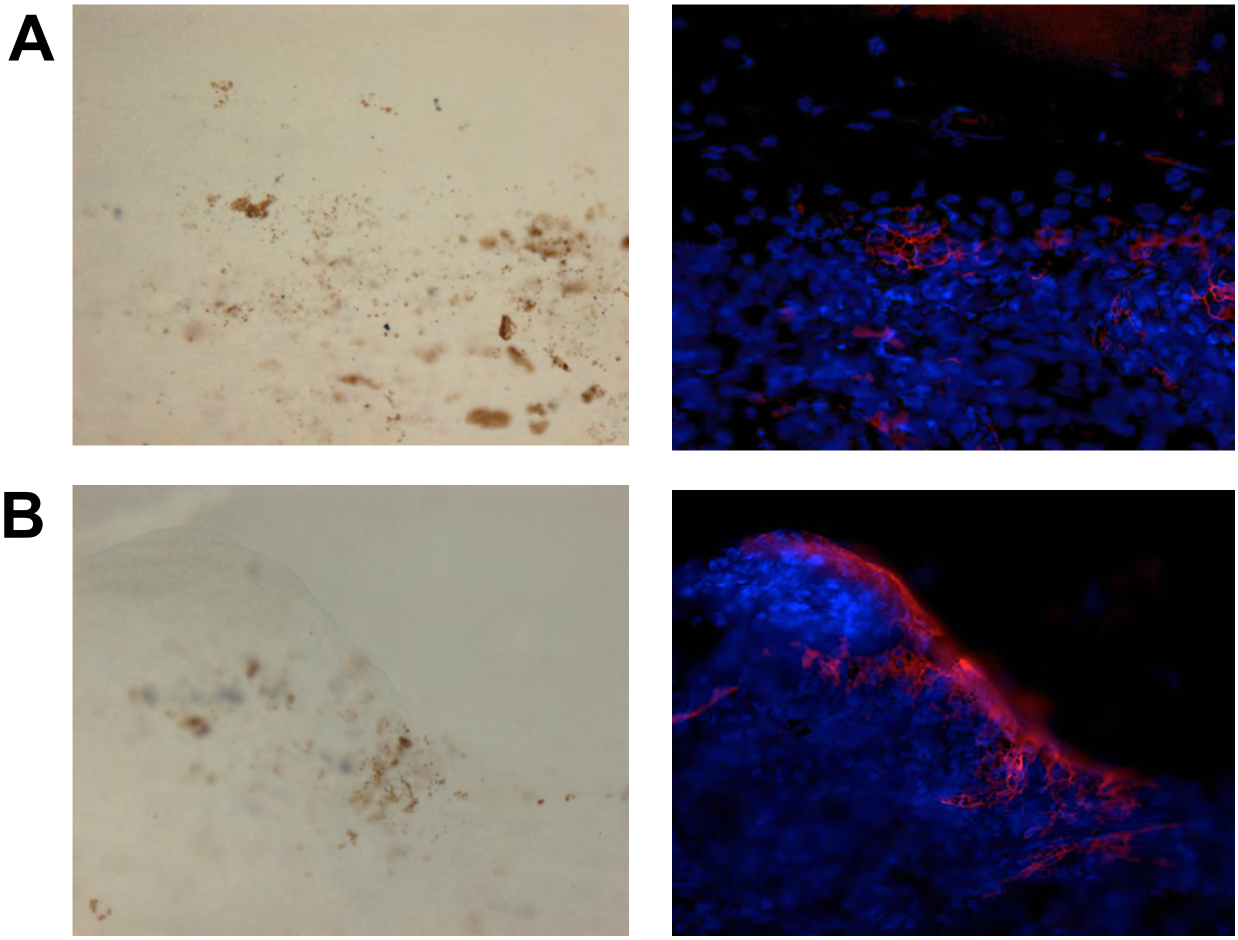
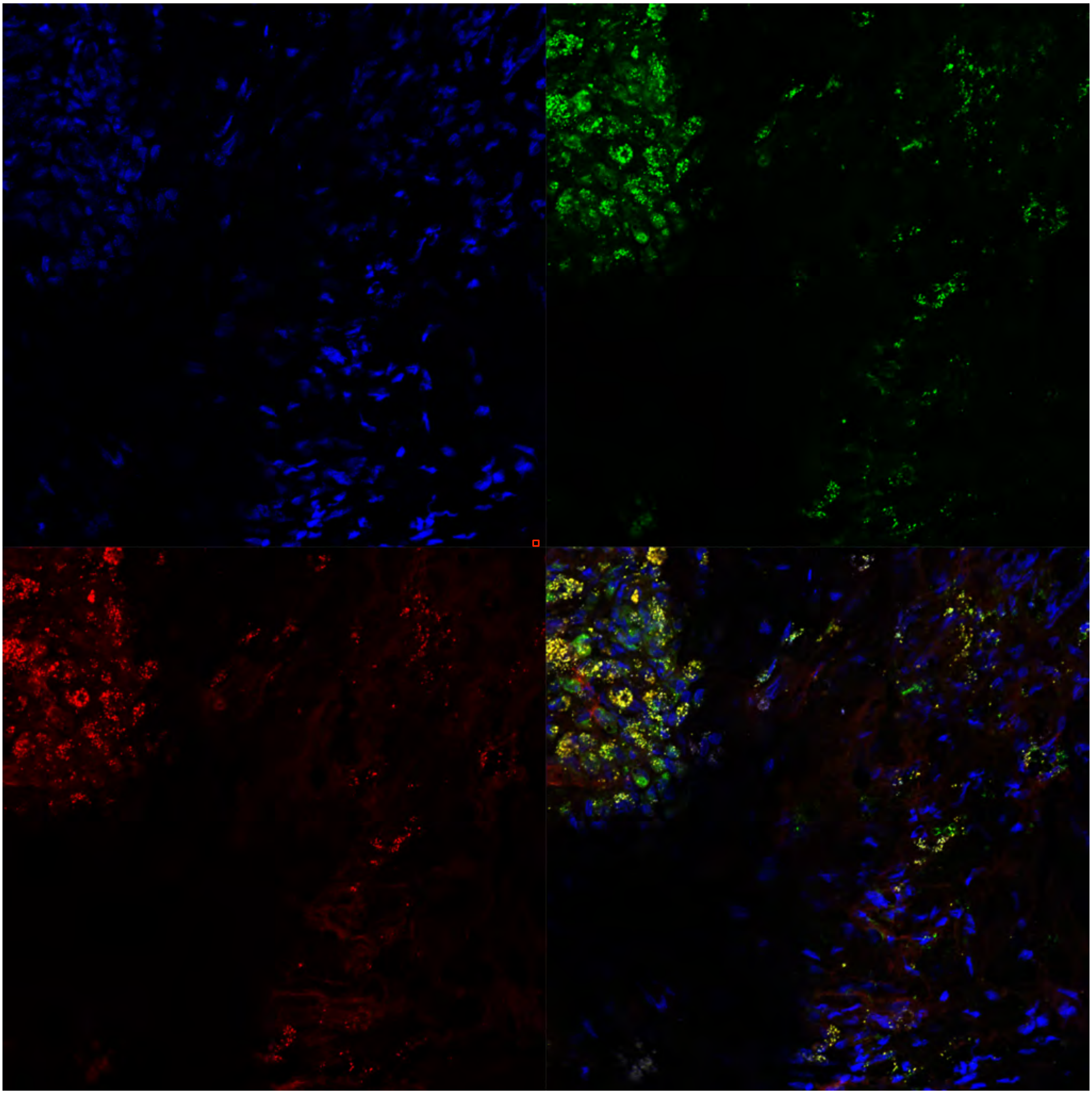


Figure 7. Two samples of human epiretinal membranes obtained at surgery from the same patient. Brightfield images (**left**) show the presence of pigmented cells, most likely RPE cells, RPE derivatives. Immunofluorescence images (**right**) show the highly cellular nature of the membrane by DAPI staining for nuclei (blue). In addition, epiretinal membranes display cell-associated anti-A2 immunoreactive material (**red**), at the vitreal border and within the lesion. Original magnification 200x.

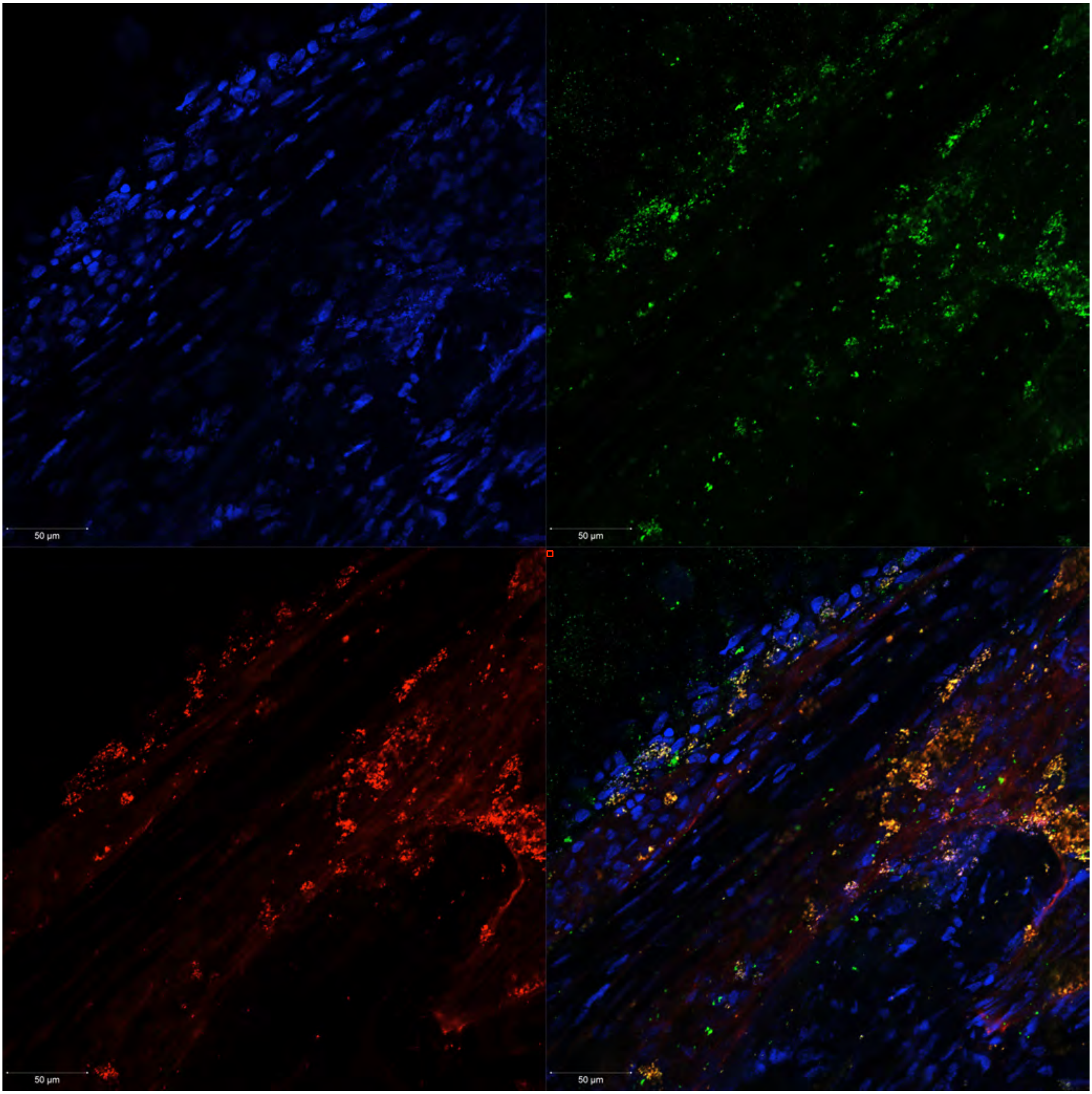
Figure 9B



Merge

Figure 8B. Immunofluorescence staining of sections through a human epiretinal membrane obtained at. Confocal images show nuclear staining (DAPI, blue), staining for macrophages (CD68, green), and staining for annexin A2 (red). Lower right panel shows co-localization of red and green signals in some groups of cells, indicating the presence of A2-expressing monocytes, macrophages, or microglia. . Original magnification 250x. Sample HS685.

Figure 9C



Merge

Figure 8B. Immunofluorescence staining of sections through a human epiretinal membrane obtained at. Confocal images show nuclear staining (DAPI, blue), staining for RPE cells (RPE65, green), and staining for annexin A2 (red). Lower right panel shows co-localization of red and green signals in some groups of cells, indicating the presence of A2-expressing cells of the RPE lineage. Original magnification 250x.. HS685.